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BINDING OF ALPHA-BUNGAROTOXIN TO SINGLE IDENTIFIED NEURONS OF --ETC(U)  
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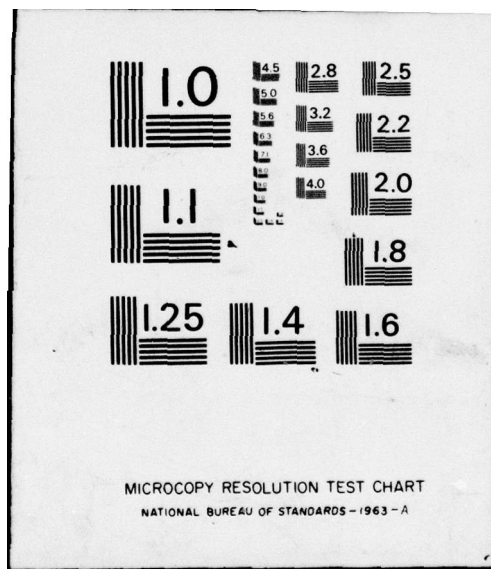
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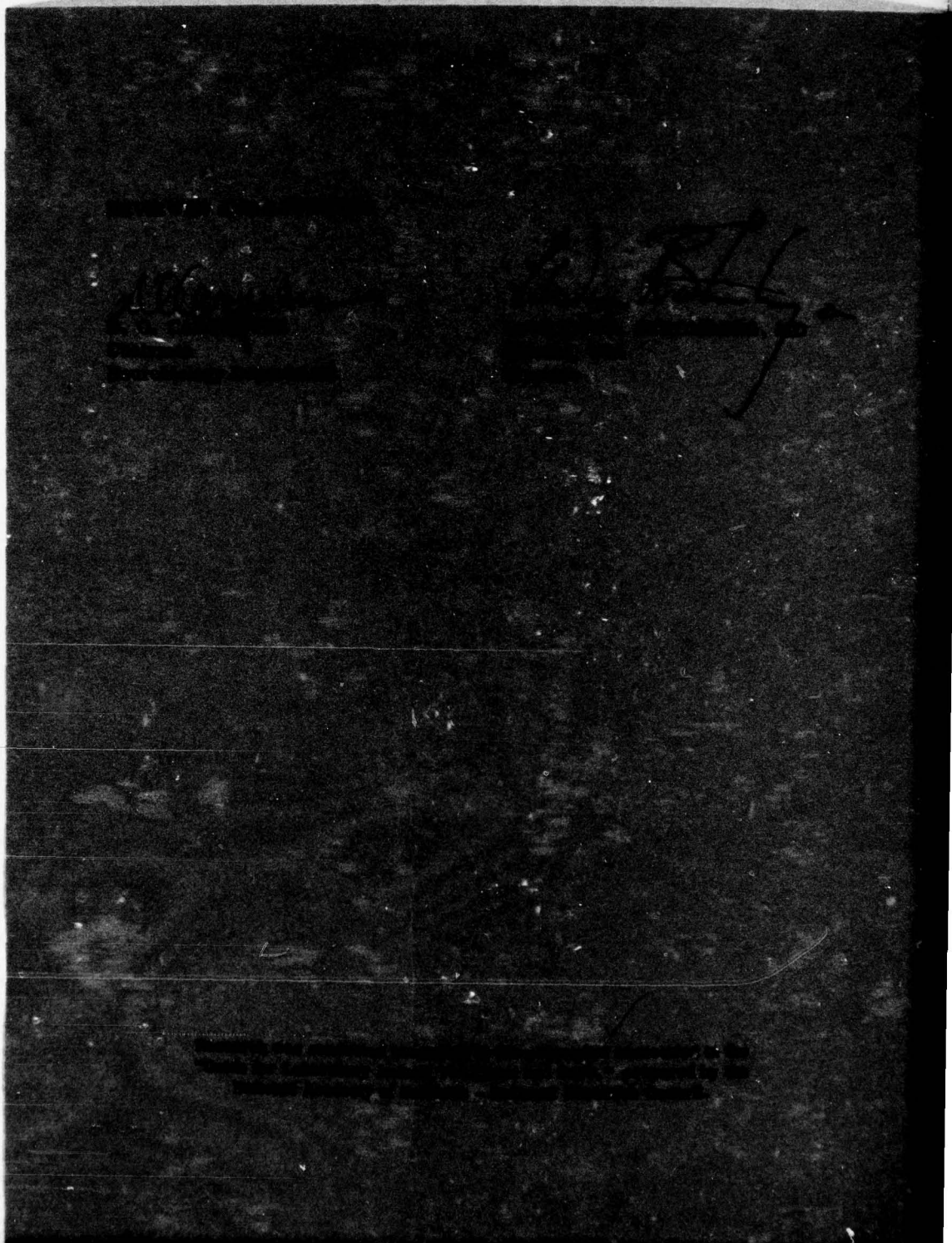
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## INTRODUCTION

There are three different responses to acetylcholine in the nervous system of the marine mollusc Aplysia resulting from conductance increases to  $\text{Na}^+$ ,  $\text{Cl}^-$ , and  $\text{K}^+$ , respectively.<sup>10</sup> These three responses are pharmacologically distinguishable, in that the  $\text{Na}^+$  response is inhibited by hexamethonium, the  $\text{Na}^+$  and  $\text{Cl}^-$  responses by curare, and the  $\text{K}^+$  response by tetraethylammonium.<sup>10</sup> This preparation is particularly well suited for a comparison of the electrophysiological and biochemical properties of the acetylcholine receptor for a number of reasons. (1) Many individual nerve cell bodies are large (100-800  $\mu\text{m}$  in diameter) and can be easily isolated for biochemical and electrophysiological analysis. (2) Some of these single neurons are sufficiently distinctive in size, color, and position in the ganglia to be visually identifiable in every preparation. (3) The identified neurons always have the same ionic response(s) to acetylcholine from one preparation to the next, and the response(s) and pharmacologic sensitivities for these responses are known for many neurons. (4) Although not the site of natural synapses the soma of all the neurons is covered with acetylcholine receptors which appear to be indistinguishable from those at natural synapses.<sup>11</sup>

Snake toxins, such as  $\alpha$ -bungarotoxin, have proven valuable tools for elucidating the biochemistry and pharmacology of the nicotinic acetylcholine receptor in brain,<sup>2</sup> electric organ,<sup>13,17</sup> and frog and mammalian neuromuscular junction.<sup>14</sup> Although in Aplysia all three types of response to acetylcholine differ from classical nicotinic responses, all are blocked by  $\alpha$ -bungarotoxin. Furthermore, the concentration of [ $^{125}\text{I}_2$ ]  $\alpha$ -bungarotoxin binding sites in a crude ganglionic homogenate is sufficient (approximately 25 pmoles/mg protein) to suggest that binding assays can be performed on single, identified neurons.<sup>21</sup>

The present studies were begun in an attempt to elucidate whether or not the three acetylcholine responses of Aplysia neurons reflect three different receptors on the basis of characteristics of [ $^{125}\text{I}_2$ ]  $\alpha$ -bungarotoxin binding to single identified neurons. In addition we have begun to study the ability of those pharmacologic agents which block only one of the three physiologic responses,

e.g., hexamethonium, to inhibit [ $^{125}\text{I}_2$ ]  $\alpha$ -bungarotoxin binding. We find that the apparent dissociation constant for toxin binding is the same for all neurons studied and that hexamethonium is no less effective in blocking binding to  $\text{Cl}^-$  and  $\text{K}^+$  neurons than  $\text{Na}^+$  neurons.

## METHODS

Aplysia dactylomela were collected from waters around Bermuda and maintained until use in flowing natural seawater. After dissection the cerebral, left pleural, and abdominal ganglia were pinned to a Sylgard (Dow Corning) filled dissecting chamber. Neurons were identified by size, color, and location using the criteria of Frazier et al.<sup>8</sup> for abdominal neurons, the descriptions of Weinreich et al.<sup>25</sup> for the giant cerebral neuron ( $\text{C}_1$ ), and Hughes and Tauc<sup>9</sup> for the left pleural giant cell (PGC). Because electrophysiological confirmation of neuronal identification was not made it is likely that some cells were not correctly identified. This is especially true for neurons  $\text{L}_7$  and  $\text{L}_{11}$ . Neuron  $\text{R}_{15}$  in A. dactylomela was consistently more yellow, less white, than in A. californica and may have occasionally been mistaken. Cells  $\text{R}_2$ ,  $\text{R}_{14}$ ,  $\text{L}_{2-6}$ ,  $\text{C}_1$ , and PGC were usually not questionable and were not dissected unless identification was certain. Single cells were dissected as previously described,<sup>18</sup> with care taken to minimize contamination by small adherent neuronal cell bodies.

In order to estimate the density of receptor sites on given cells the greatest and smallest diameters were measured at the time of dissection. The average dimensions did not differ significantly from those reported by Zeman and Carpenter.<sup>26</sup> Surface areas were calculated using an equation for oblate spheroids.<sup>20</sup>

The cell bodies of individual identified cells were pooled in 100  $\mu\text{l}$  of Millipore-filtered seawater<sup>21</sup> containing 2 mg/ml bovine serum albumin in a microhomogenizer. After homogenization the samples were frozen in a final volume of 200  $\mu\text{l}$ . Homogenates of pleural, pedal, buccal, cerebral and abdominal ganglia were prepared as previously described<sup>21</sup> for comparison with

homogenates of single neurons. All subsequent dilutions of samples were made in Millipore-filtered seawater containing 2 mg/ml bovine serum albumin.

On the day the binding experiments were performed the homogenates were thawed, an aliquot taken to determine the approximate concentration of toxin binding material, and the original homogenate diluted to an appropriate volume to permit the maximal number of assays while maintaining sufficient binding activity. Assays were performed by adding aliquots of homogenate, inhibitor or filtered seawater, where appropriate, and [ $^{125}\text{I}_2$ ]  $\alpha$ -bungarotoxin to a final volume of 250  $\mu\text{l}$ . Incubations were made for 90 minutes at 22 $^{\circ}$ -24 $^{\circ}\text{C}$ . Previous studies with a ganglionic homogenate have shown this to be sufficient time to reach steady-state conditions.<sup>21</sup> After incubations the reaction mixture was collected on Millipore filters (type EGWP), washed, and counted as previously described.<sup>21</sup> Diiodinated  $\alpha$ -bungarotoxin with a specific activity of 320-400 Ci/nM was prepared as described by Vogel et al.<sup>24</sup> Kinetic parameters were obtained using an unweighted linear regression analysis for Scatchard plots.

## RESULTS

In order to determine the nature of the [ $^{125}\text{I}_2$ ]  $\alpha$ -bungarotoxin binding site on neurons with different acetylcholine responses, homogenates of either pooled single identified neurons or, as in the case of neurons L<sub>2-6</sub>, pooled clusters of neurons with similar acetylcholine responses were incubated with varying concentrations of toxin. Data from neurons representing Na<sup>+</sup> (R<sub>15</sub>), Cl<sup>-</sup>, and K<sup>+</sup> (PGC and L<sub>2-6</sub>), and K<sup>+</sup> (R<sub>14</sub>) responses are shown in Figure 1. The binding to all cells was saturable while the apparent maximum binding per cell was different for each neuron. Kinetic parameters of binding were determined from these and similar data for eight neurons using Scatchard plots (Figure 2). The intercept on the abscissa represents the number of toxin binding sites per cell and the slope of the line is the negative value of the apparent dissociation constant. There appears to be no significant difference in the apparent dissociation constants for these neurons, although there are significant differences in the

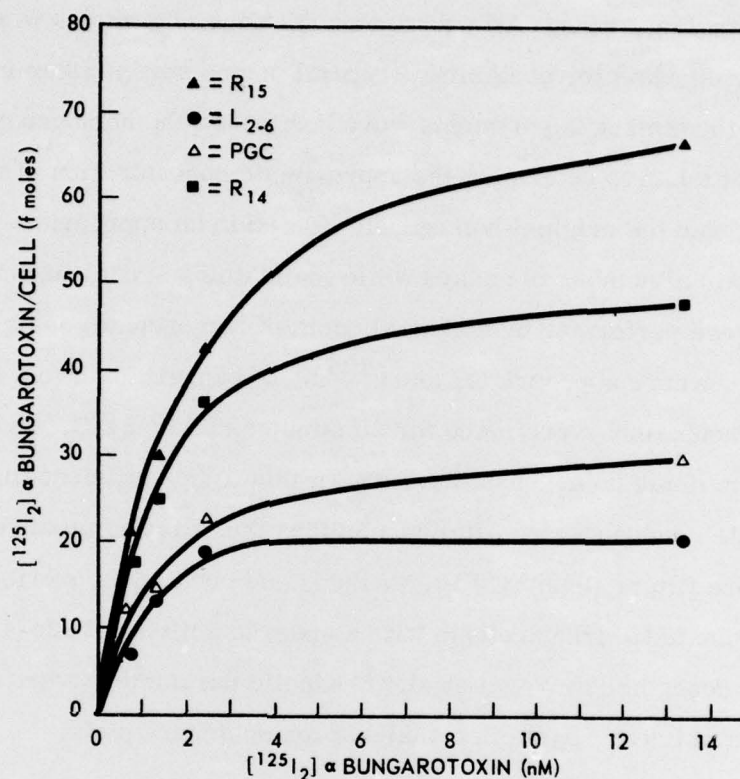


Figure 1. [<sup>125</sup>I]<sub>2</sub> α-bungarotoxin binding to single, identified neuron homogenates. The ordinate is the total toxin bound in f moles while the abscissa is the concentration (nM) of toxin in the incubation medium.

numbers of toxin binding sites per cell. These results demonstrate that although there is a quantitative difference in acetylcholine receptors per cell, qualitatively they are similar.

Table 1 lists the neurons studied, their ionic response to iontophoretically applied acetylcholine, the kinetic parameters from the Scatchard analysis, apparent dissociation constants and receptor concentration per cell, and geometric measurements to determine the number of receptors per unit of surface area ( $\mu^2$ ). The mean apparent dissociation constant is  $2.0 \pm 0.6$  (S.E.M.) nM which is not significantly different from values for entire ganglionic homogenates ( $1.2 \pm 0.4$  nM). The values for surface area were calculated from the cell

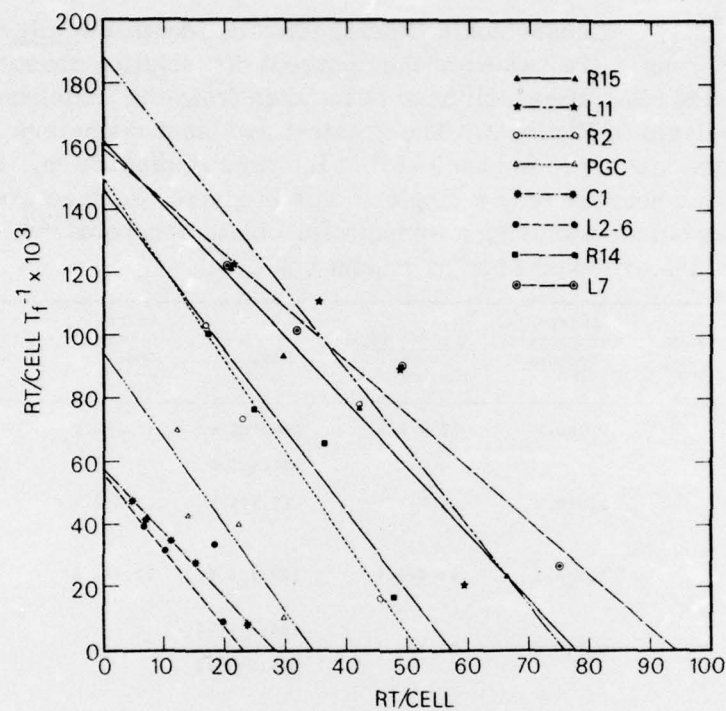


Figure 2. Scatchard analysis of  $[^{125}\text{I}_2]$   $\alpha$ -bungarotoxin binding to homogenates of pooled single, identified *Aplysia* neurons. The neuron homogenate was incubated with varying concentrations of toxin. All incubations were for 90 min. The ordinate is the ratio of the amount of toxin bound per cell (RT/cell) to the amount of free toxin in the reaction mixture, while the abscissa is the amount of toxin bound per cell (RT/cell). The intercept on the abscissa is the total number of binding sites per cell, while the negative slope of the lines give the  $K_D$ . The lines drawn represent an unweighted linear regression analysis.

diameters as described in Methods. Such values are underestimates of actual surface area since membrane infoldings on all of these neurons result in an increase of surface area of at least fivefold to tenfold.<sup>6,15</sup> Coggeshall (personal communication) has also noted that some neurosecretory cells, including  $R_{14}$ , show a several-fold greater membrane infolding than other neurons. Thus the density values in Table 1 are only crude overestimates of receptor density.

Table 1. [ $^{125}\text{I}_2$ ]  $\alpha$ -Bungarotoxin Binding Sites on Identified *Aplysia* Neurons. The values of the apparent dissociation constants and binding sites/cell have been taken from the Scatchard analysis in Figure 2. The greatest and least diameters were measured for each cell at the time of dissection. For round neurons only a single radius is given. Surface area was calculated using a formula for oblate spheroids.<sup>20</sup> Values are expressed as means  $\pm$  S.E.M.

Neuron	Ionic Conductance Change Na <sup>+</sup> Cl <sup>-</sup> K <sup>+</sup>	Apparent Dissociation Constant (nM)	Binding sites cell (fmol/cell)	Neuron Radius ( $\mu$ )	Neuron Surface Area ( $\mu^2 \times 10^4$ )	Binding sites $\mu^2 \times 10^4$
R <sub>15</sub>	+	2.1 $\pm$ 0.2	77.9 $\pm$ 3.3	148.4 $\pm$ 6.8 x 144.3 $\pm$ 6.0	27.1 $\pm$ 2.2	17.3
C <sub>1</sub>	+	2.0 $\pm$ 0.1	28.7 $\pm$ 0.7	115.3 $\pm$ 6.7	17.3 $\pm$ 2.0	10.0
L <sub>7</sub>	+	1.7 $\pm$ 0.3	93.0 $\pm$ 8.6	129.9 $\pm$ 4.9	21.4 $\pm$ 1.6	26.2
R <sub>2</sub>	+	2.8 $\pm$ 0.4	51.6 $\pm$ 3.5	286.8 $\pm$ 14.9 x 211.5 $\pm$ 9.2	70.8 $\pm$ 4.7	4.4
L <sub>11</sub>	+	2.5 $\pm$ 0.8	69.9 $\pm$ 10.6	180.5 $\pm$ 10.6	42.6 $\pm$ 4.8	9.9
PGC	+	2.7 $\pm$ 0.8	32.3 $\pm$ 4.5	263.2 $\pm$ 13.1 x 221.8 $\pm$ 9.2	70.1 $\pm$ 4.2	2.8
L <sub>2-6</sub>	+	2.4 $\pm$ 0.0	23.5 $\pm$ 0.2	155.6 $\pm$ 4.1	30.0 $\pm$ 1.7	4.6
R <sub>14</sub>	+	2.6 $\pm$ 0.5	55.6 $\pm$ 4.9	140.4 $\pm$ 11.1	24.1 $\pm$ 4.2	13.9

Assuming that the estimates of surface area are consistent for all but neurosecretory cells, e.g., R<sub>14</sub>, there appears to be a higher density of  $\alpha$ -bungarotoxin binding sites on neurons with Na<sup>+</sup> acetylcholine responses than on those with only Cl<sup>-</sup> or Cl<sup>-</sup> and K<sup>+</sup> responses. Since the surface infoldings on R<sub>14</sub>, the only pure K<sup>+</sup> cell, are greater than the other cells this neuron may also have a lower density than neurons with a Na<sup>+</sup> response. However, L<sub>11</sub>, a neuron with a Cl<sup>-</sup> response, has a receptor density similar to the Na<sup>+</sup> cells studied.

Figure 3 shows the effects of hexamethonium on toxin binding to the various single cell homogenates. Hexamethonium was as effective in blocking toxin

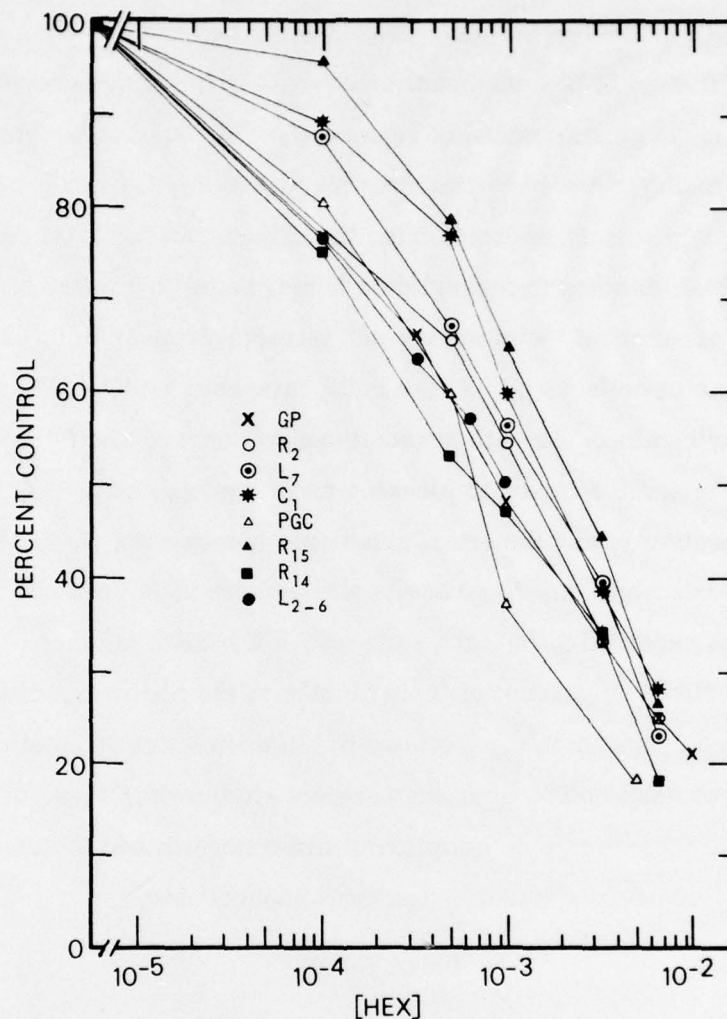


Figure 3. Inhibition of [ $^{125}\text{I}_2$ ]  $\alpha$ -bungarotoxin binding to identified neurons by hexamethonium. Toxin binding is expressed as percent of the binding in the absence of any hexamethonium. Concentrations ( $\times 10^{-3}$  M) of hexamethonium giving 50 percent inhibition of toxin binding are 0.89 (GP = ganglionic preparation), 1.3 ( $R_2$ ), 1.6 ( $L_7$ ), 1.7 ( $C_1$ ), 0.68 (PGC), 2.4 ( $R_{15}$ ), 0.75 ( $R_{14}$ ) and 1.0 ( $L_{2-6}$ ).

binding in neurons with  $\text{Cl}^-$  or  $\text{K}^+$  responses as it was on neurons with only a  $\text{Na}^+$  response. Although the concentration of hexamethonium required to block 50 percent of toxin binding had a range of sensitivities (0.68-2.4 mM), there

was no correlation between the ionic response characteristic of a given neuron and the concentration of hexamethonium required to block 50 percent of toxin binding. The mean concentration of hexamethonium required to inhibit 50 percent of toxin binding in the identified neurons was  $1.2 \pm 0.4$  mM. This concentration is not significantly different from the concentration required to inhibit 50 percent of toxin binding to a ganglionic homogenate (0.9 mM).

Similar experiments with curare and tetraethylammonium were also made on a homogenate of cells L<sub>2-6</sub>. These cells have both a Cl<sup>-</sup> and K<sup>+</sup> ionic response to acetylcholine. As with hexamethonium, curare and tetraethylammonium showed the same efficacy in blocking toxin binding on L<sub>2-6</sub> (6.7  $\mu$ M and 1.6 mM, respectively) and the crude ganglionic homogenate (7.6  $\mu$ M and 2.0 mM, respectively). Similar results with curare have been obtained for cell R<sub>2</sub> with 6.9  $\mu$ M required to inhibit 50 percent of the toxin binding. Tetraethylammonium inhibited 50 percent of toxin binding in the pleural giant cell at 2.0 mM and in L<sub>11</sub> at 3.2 mM. Because of the difficulties in obtaining an adequate quantity of tissue other neurons were not studied with these two drugs. Thus with all of these drugs no significant differences in sensitivity were found between identified neurons and the ganglionic homogenate.

#### DISCUSSION

There are three important conclusions from these experiments: (1) Neurons which may have any of three different ionic, and pharmacologically distinguishable, responses have only a single class of [<sup>125</sup>I<sub>2</sub>]  $\alpha$ -bungarotoxin binding sites. (2) Hexamethonium, which inhibits only the Na<sup>+</sup> response in electrophysiologic studies, inhibits [<sup>125</sup>I<sub>2</sub>]  $\alpha$ -bungarotoxin binding with equal efficacy to neurons of all three classes. (3) The density of acetylcholine receptors appears to be greater on the Na<sup>+</sup> neurons than others.

These interpretations are predicated upon the assumption that neurons were properly identified and isolated without contamination. There are several possible sources of error. The first is the uncertainty of neuron identification

in the absence of electrophysiological confirmation, especially for L<sub>7</sub>, L<sub>11</sub>, and R<sub>15</sub>. The identity of most other neurons was rarely in question. Another problem is that small neurons which are sometimes not visible under the dissecting microscope may adhere to the larger neuronal cell bodies. Although there is certainly some contaminations from these sources it is very unlikely that this is adequate to significantly influence the results, since all neurons were dissected as carefully as possible to minimize such contamination. Finally there is a glial investment around all neurons. It is possible that such cells might contain acetylcholine receptors similar to those described by Villegas on the glial cells around squid axons.<sup>23</sup> However a number of observations suggest the [<sup>125</sup>I<sub>2</sub>]  $\alpha$ -bungarotoxin binding we are studying is directly associated with neuronal acetylcholine receptors. (1) The concentration of toxin required to block the acetylcholine responses is similar to the amount of toxin required for saturable binding.<sup>21</sup> (2) The time course of the dissociation of toxin binding and the return of the physiological response after treatment with  $\alpha$ -bungarotoxin are similar (approximately 45 minutes).<sup>21</sup> (3) Both single cells and a homogenate of ganglia show a single apparent dissociation constant suggesting a single class of receptors.

[<sup>125</sup>I<sub>2</sub>]  $\alpha$ -bungarotoxin binding. There are several indications that  $\alpha$ -bungarotoxin interacts specifically with the acetylcholine binding site. (1) Under the conditions of our experiments (A. dactylorella in the summer)  $\alpha$ -bungarotoxin blocks all three responses.<sup>21</sup> (2) Carbamylcholine, a stable cholinergic agonist, interacts with  $\alpha$ -bungarotoxin as a linear competitive inhibitor<sup>4</sup> using the analysis and nomenclature described by Cleland,<sup>5</sup> suggesting that the binding of these two substances is to the same site. (3) Greater than 95 percent of toxin binding is inhibited by curare and all binding of [<sup>125</sup>I<sub>2</sub>]  $\alpha$ -bungarotoxin is inhibited by unlabeled toxin, indicating very little nonspecific toxin binding.<sup>21</sup> (4)  $\alpha$ -Bungarotoxin has little effect on acetylcholinesterase even at concentrations as high as 1.0 M.<sup>4</sup> Thus it appears likely that  $\alpha$ -bungarotoxin binds specifically to the acetylcholine receptor of neurons.

Pharmacology of  $\alpha$ -bungarotoxin binding. The observation that hexamethonium inhibits [ $^{125}\text{I}_2$ ]  $\alpha$ -bungarotoxin binding to all identified neurons with equal potency is surprising since it blocks the response to acetylcholine in only neurons with the  $\text{Na}^+$  response. This result suggests that hexamethonium has a binding site on all neurons but can only block the conductance change to  $\text{Na}^+$ . Although curare and tetraethylammonium were not studied in detail, it appears that these drugs also have binding sites on all neurons, since all three drugs inhibit toxin binding to a membrane homogenate containing all three types of neurons<sup>21</sup> and were equally potent in inhibiting toxin binding to the single neurons studied.

Kehoe et al.<sup>12</sup> have recently reported failure to confirm our original observation<sup>21</sup> that  $\alpha$ -bungarotoxin blocks all three ionic responses to acetylcholine. They find only the  $\text{Cl}^-$  response sensitive and then only at  $10^{-5}$  M  $\alpha$ -bungarotoxin. We find electrophysiological results in total agreement with their observations in both *Aplysia californica* and *A. dactylomela* in all but the summer, and much less sensitivity of *A. californica*, even in summer (Gaubatz and Carpenter, unpublished). In addition, there is little or no binding of [ $^{125}\text{I}_2$ ]  $\alpha$ -bungarotoxin to ganglionic homogenates of either species in fall, winter, or spring (Shain, Kebabian and Carpenter, unpublished). We conclude that the apparent discrepancy with Kehoe et al.<sup>12</sup> reflects a seasonal and species variation in sensitivity to the toxin. The demonstration of binding of [ $^{125}\text{I}_2$ ]  $\alpha$ -bungarotoxin to single identified neurons which show all three responses is further evidence that, under the conditions of our experiments, the toxin does act on all three types of response.

Receptor density. The calculated densities of acetylcholine receptors shown in Table 1 are higher than those reported for either the neuromuscular junction<sup>7</sup> or the postsynaptic membrane of electroplax<sup>3</sup> ( $3.4 \times 10^4$  and  $3.3 \times 10^4$  receptors/ $\mu\text{m}^2$ , respectively). However as detailed in Results the density values presented for the *Aplysia* neurons are overestimates due to infoldings of the neuronal cell surface. If density values are recalculated

considering the additional surface area, the values for all cells would be fivefold to tenfold less. To obtain estimates of the surface area for the neurosecretory cell R<sub>14</sub>, which is even more highly convoluted, an additional dilution of the receptor density by at least fivefold must be considered. Table 2 compares density values calculated using geometric (oblate spheroid) criteria and adjusted values considering additional membrane area due to infoldings. The highest adjusted values for receptor density are similar to those at the neuromuscular

Table 2. Acetylcholine Receptor Densities on Various Tissues and Single Neurons

				Average Geometric Density/ $\mu\text{m}^2$	Correction Factor	Corrected Receptor Density
Neuromuscular junction						
Miledi and Potter, <sup>14</sup> frog				$10 \times 10^4$		
Porter et al., <sup>16</sup> mouse				$1.2 \times 10^4$		
Salpeter & Eldefrawi, <sup>19</sup> mouse				$0.7 \times 10^4$		
Fertuck & Salpeter, <sup>7</sup> mouse				$0.7 \times 10^4$	4-6	$2.8-4.2 \times 10^4$
Barnard et al., <sup>1</sup> mouse & bat				$0.88 \times 10^4$		$2.0-2.5 \times 10^4$
Electroplax						
Bourgeois, et al. <sup>3</sup>				$3.3 \times 10^4$		
<u>Aplysia</u> neurons						
cell	Na	Cl	K			
R <sub>15</sub>	+			$17.3 \times 10^4$	0.1-0.2	$1.7-3.5 \times 10^4$
C <sub>1</sub>	+			$10.0 \times 10^4$	0.1-0.2	$1.0-2.0 \times 10^4$
L <sub>7</sub>	+	+		$26.2 \times 10^4$	0.1-0.2	$2.6-5.2 \times 10^4$
R <sub>2</sub>		+		$4.4 \times 10^4$	0.1-0.2	$0.4-0.9 \times 10^4$
L <sub>11</sub>		+		$9.9 \times 10^4$	0.1-0.2	$1.0-2.0 \times 10^4$
PGC		+	+	$2.8 \times 10^4$	0.1-0.2	$0.3-0.6 \times 10^4$
L <sub>2-6</sub>		+	+	$4.6 \times 10^4$	0.1-0.2	$0.5-0.9 \times 10^4$
R <sub>14</sub>			+	$13.9 \times 10^4$	0.02-0.04	$0.5-1.0 \times 10^4$

junction and in electroplax. It is interesting to note that cells with  $\text{Na}^+$  responses have higher receptor densities than cells with  $\text{Cl}^-$  or  $\text{Cl}^-$  and  $\text{K}^+$  responses, except for  $\text{L}_{11}$ . The functional significance of this is not clear; however, experimentally, these differences may be seen as differences in sensitivity to  $\alpha$ -bungarotoxin since at least five times more toxin would probably be required to completely inhibit observed acetylcholine responses. Thus, the receptor density in A. dactylomela neurons varies from neuron to neuron but may approach values calculated for neuromuscular junction and electroplax.

Functional organization of acetylcholine receptors. Swann and Carpenter<sup>22</sup> found that for dopamine, as well as acetylcholine, there were at least three ionic responses, resulting from  $\text{Na}^+$ ,  $\text{Cl}^-$  and  $\text{K}^+$  conductance increases. The temporal and thermal characteristics were identical for the same ionic responses caused by different transmitters. They have interpreted these results in terms of a model of receptor organization in which any receptor (i.e., the transmitter binding site) can be associated with any of three ionophores (i.e., conductance increase mechanisms). This model suggests that the transmitter binding site is identical for responses associated with any of the three conductance changes, and that the ionophores for a given ionic conductance are identical when associated with different receptors. The present results support this model by showing that when  $[^{125}\text{I}_2]$   $\alpha$ -bungarotoxin binding is used to monitor acetylcholine binding sites there are no significant differences in apparent dissociation constants among the neurons studied or between the identified neurons and the ganglionic homogenate. There appears to be a single class of acetylcholine binding sites.

The results with hexamethonium suggest that all acetylcholine receptor complexes in Aplysia have hexamethonium binding sites even though it inhibits only the  $\text{Na}^+$  response. The model suggests that the receptor consists of a transmitter binding site moiety and an ionophore moiety. The observation that hexamethonium inhibits only the  $\text{Na}^+$  response but blocks  $\alpha$ -bungarotoxin binding to all cells must mean either (1) that hexamethonium binds to the receptor

moiety, but not at the acetylcholine binding site, and in such a way as to interfere only with activation of the  $\text{Na}^+$  conductance, or (2) that hexamethonium binds to the ionophore moiety but the ionophore moiety has the potential to mediate all three ionic responses. Since hexamethonium does not appear to block the  $\text{Na}^+$  or any response to other neurotransmitters than acetylcholine, the first possibility seems the more likely.

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